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·综述·

人诱导多能干细胞成骨分化的研究进展

廖凌子[1]，　宋雅朦[1]，　刘美萱[1]，　李思怡[1]，　周平[1,2]
1. 兰州大学口腔医学院，甘肃　兰州（730000）；　2. 甘肃省牙颌面重建与生物智能制造重点实验室，甘肃　兰州（730000）

【摘要】 骨骼疾病如骨质疏松、骨关节炎等已成为亟待解决的人类健康问题，细胞治疗及组织工程技术被认为是理想的治疗方法之一。人诱导多能干细胞（human induced pluripotent stem cells，hiPSCs）具备体外长期自我更新和分化所有三胚层来源体细胞的独特功能，已成为目前最有前景的成骨细胞来源。因此，需要构建成分明确的hiPSCs体外成骨向诱导分化体系，获得符合临床应用要求的成骨样细胞。许多团队在促进hiPSCs向成骨分化成熟的直接路径和经间充质干细胞的间接路径方面取得了实质性的进展，本文针对这两类成骨分化路径及其应用现状进行综述，以期为骨再生技术提供参考。现有研究借助拟胚体法和单层诱导法，基于生物材料，构建可支持hiPSCs体外培养和成骨向诱导分化体系。然而，目前的研究主要存在成分不明确，分化效率低等局限，基于特定化合物严格调控的分阶段式和三维定向诱导体系是未来的主要研究方向。

【关键词】 骨再生；　人诱导多能干细胞；　成骨细胞；　诱导分化；　拟胚体法；　单层诱导法；　分阶段诱导法；　三维定向；　诱导体系
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Advances in the differentiation of human induced pluripotent stem cells into osteoblasts

LIAO Lingzi[1], SONG Yameng[1], LIU Meixuan[1], LI Siyi[1], ZHOU Ping[1,2].　1. School of Stomatology, Lanzhou University, Lanzhou 73000, China;　2. Gansu Provincial Key Laboratory of Dental and Maxillofacial Reconstruction and Biological Intelligent Manufacturing, Lanzhou 73000, China
Corresponding author: ZHOU Ping, Email: zhoup@lzu.edu.cn, Tel: 86-18293181781

【Abstract】 Bone diseases, such as osteoporosis and osteoarthritis, have emerged as pressing public health concerns requiring immediate attention and resolution. Cellular therapy and tissue engineering techniques are among the most promising therapeutic approaches for such conditions. Human induced pluripotent stem cells (hiPSCs) possess remarkable capacity for indefinite self-renewal *in vitro* and the ability to differentiate into all somatic cell types originating from the three germ layers, thereby making them a promising source of osteoblasts. Consequently, it is crucial to establish a well-delineated system for osteogenic differentiation of hiPSCs in vitro, with the aim to generate osteoblast-like cells that conform to clinical application standards. Numerous research teams have achieved substantial advancements in both the direct osteogenic differentiation of hiPSCs and the indirect pathway via mesenchymal stem cells. In this article, we provide a comprehensive review of these two osteogenic differentiation pathways and their current applications, with the aim of serving as a valuable reference for bone regeneration technologies. Current research efforts have relied on embryoid body formation and monolayer induction methods utilizing biomaterials to develop a system that facilitates in vitro culture and osteogenic differentiation of hiPSCs. However, the existing research is primarily constrained by unclear system components and low efficiency. Therefore, the development of a stepwise and three-dimensional induction system based on stringent regulation by specific compounds is a primary research direction for the future.

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【作者简介】廖凌子，本科，Email：liaolz20@lzu.edu.cn
【通信作者】周平，教授，博士，Email：zhoup@lzu.edu.cn，Tel：86-18293181781

【Key words】 bone regeneration; human induced pluripotent stem cells; osteoblast; induced differentiation; embryoid bodies method; monolayer culture method; defined stepwise differentiation method; three-dimensional orientation; induction system
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肿瘤、创伤和先天畸形引起的骨组织丧失通常采用移植技术进行修复，但在临床实践中，存在组织来源有限、二次手术、免疫原性和伦理道德等诸多问题[1]。近年来，组织工程技术的快速发展实现了骨、软骨和肌腱等多种器官的再生，为临床治疗带来了新思路[2-3]。在骨髓中发现的间充质干细胞（mesenchymal stem cells，MSCs）已显示出分化为成骨细胞（osteoblasts，OBs）的宝贵潜力，但人体来源的MSCs存在取材不便和容易老化等缺点，限制了其应用[4-5]。

OBs也可由人多能干细胞（human pluripotent stem cells，hPSCs）分化而来[6-7]。hPSCs包括人诱导多能干细胞（human induced pluripotent stem cells，hiPSCs）和人胚胎干细胞（human embryonic stem cells，hESCs）[8]。hiPSCs是由人体细胞经重编程技术编辑得到的一类高度未分化细胞，因其良好的自我更新与多向分化潜能引起骨修复领域的广泛关注[9-10]。hiPSC-OBs有效克服了现有不足，其体外扩增能力强、来源广泛且无免疫原性，具有重要的科学意义和临床价值。目前开发出的hiPSC-OBs培养体系可以较好地模拟骨骼微环境，在成骨诱导过程中能复制体内骨组织的结构、功能和遗传特征[11]。然而，建立体外诱导分化体系，以便在无异种成分条件下获得足够数量的OBs仍面临着许多挑战[12]。本文就hiPSCs的最新成骨路径及其在骨再生医学中的应用现状进行综述。

1　成骨细胞的体内发育过程及调控

骨骼的发育是由基因网络和信号系统相互调控的过程[13]。深入理解骨骼发育过程中的关键基因和转录因子、修复过程中不同细胞的功能和信号通路，是探索hiPSCs成骨向分化的前提[14]。

OBs是参与成骨最主要的细胞，其可分泌有机骨基质促矿化，维持骨稳态[15]。OBs生成障碍或功能失调可引起骨骼微结构破坏，骨形成缺陷，导致骨代谢疾病[16]。大量的分泌因子和转录因子已被确定可调节成骨发生[17-18]。转录因子性别决定区Y框蛋白-9（sex determining region Y box protein 9，SOX9）、Runt相关转录因子2（Runt-related transcription factor 2，Runx2）和Osterix（OSX）的先后表达，分别参与了骨祖细胞、前成骨细胞及OBs的成熟[19]。成骨分化还受到多种基因的调控[20]。脑和肌肉ARNT样蛋白1（brain and muscle ARNT-like 1，BMAL1）基因作为昼夜生物钟的核心组成部分，在多种信号通路介导下参与并调控体内各项生理活动[21]。BMAL1基因的失活抑制软骨细胞和OBs并促进破骨细胞分化，阻碍软骨内成骨、膜内成骨及骨代谢，造成颌骨发育畸形、骨关节炎及骨质疏松等疾病[22]。另外，OBs谱系的分化成熟还涉及Notch、Hedgehog、骨形态发生蛋白（bone morphogenetic protein，BMP）-SMAD、Wnt/β-catenin、促分裂原活化蛋白激酶（mitogen-activated protein kinase，MAPK）、RhoA/ROCK、转化生长因子-β（transforming growth factor-β，TGF-β）等信号通路[23-30]。

2　经hiPSC-MSCs诱导分化获得成骨细胞

MSCs具有生成OBs的关键能力，但实际应用中却遇到了MSCs来源不足的问题[31-32]。由hiPSCs分化而来的MSCs可作为一种替代的细胞来源[33-34]。最早的研究利用拟胚体（embryoid bodies，EBs）将hiPSCs诱导为MSCs。在体内胚胎发育过程中，来自内部细胞的中胚层和外胚层细胞进一步分化为MSCs，而这一过程可通过将EBs诱导附着在培养板表面来模拟。之后在地塞米松、β-甘油和抗坏血酸三种因子的作用下，MSCs可实现高效的成骨诱导[35]。在MSC维持培养基（添加胎牛血清的基础培养基）中，这些细胞迅速呈现成纤维细胞形态，流式细胞术和体外分化实验显示其特异性表面标志物和成骨潜能[36]。虽然，EBs法可模拟体内MSCs的自发分化过程，但其效率低、过程不可控，并可能因残留hiPSCs而导致畸胎瘤的风险。轴旁中胚层诱导法的发展初步解决了上述问

题。在含有Wnt和BMP调节因子条件下培养hiPSCs，观察到hiPSCs快速上调轴旁中胚层标记物。在加入成骨诱导添加剂后，检测到成骨基因的表达和矿化细胞外基质的增高[37]。然而，上述方法均使用了含有滋养层和血清的诱导培养基。为了进一步促进分化并剔除血清成分，有必要在特定阶段添加功能化合物（生长因子、小分子抑制剂或维生素等），构建分步诱导体系。Kanke等[38]通过添加全反式维A酸（retinoic acid，RA），诱导hiPSCs分化为中胚层祖细胞。Hedgehog和BMP通路被证实可诱导该细胞的成骨向分化。化合物的不同成分和浓度组合还可以影响分化效率、细胞纯度以及OBs的生物学特性等[39-41]。同时，物理条件的改变，如低强度脉冲超声可促进hiPSC-MSCs的成骨潜力，但无法简化iPSC-EB-MSCs分化过程[42]。可见，初期研究侧重于自发分化过程，后续方法则更具针对性，通过调控细胞内信号通路、基因表达和细胞周期，实现对分化命运的精细调控，大大提高了细胞的分化效率。

3　hiPSCs直接分化为成骨细胞的体系构建

通过hiPSC-MSCs培育OBs仍存在效率问题，而直接将hiPSCs诱导为OBs可绕过MSCs这一中间细胞，大大降低细胞获取的成本。

同样地，研究人员在最初的直接成骨分化过程中也运用了EBs法，并通过优化分化条件、基因编辑技术以期建立高效的分化模型。Bourne等[43]将hPSC-EBs直接培养于成骨诱导培养基，检测到成骨基因的快速诱导作用及骨结节的形成和基质矿化，表明成骨诱导培养基可用于hiPSCs的直接成骨向诱导。本课题组的研究证实，通过EB法进行成骨向诱导分化时，EBs悬浮3～5 d钙盐沉积量最多，成骨分化效率较高[44]。对氧含量、机械应力和表面粗糙度等一些物理和化学参数的优化也可以加速成骨进程。缺氧可以促进EBs的悬浮和成熟，从而增强其黏附和增殖性能而不影响其分化为三胚层的多能性[45]。Manokawinchoke等[46]证明了间歇性压力可减少EBs的凋亡，其中，TGF-β信号通路可能参与了间歇性压力诱导的细胞周期进程。Limraksasin等[47]发现，0.5 Pa时的剪切应力通过Cx43和下游的Erk1/2信号增强了hiPSCs的成骨分化。由于细胞具有类似于MSCs的黏附力，多种生物支架材料被应用于促进hiPSC-EBs的成骨分化。Tahmasebi等[48]将microRNA载入静电纺丝聚己内酯（PCL）纳米纤维，实现了悬浮4~5 d的hiPSC-EBs的成骨向诱导。Kato等[49]将外周血来源的hiPSCs诱导为OBs并移植到含胶原海绵支架的大鼠颅骨缺损模型中，获得良好影像学预后，并未发现畸胎瘤的形成。新形成的骨骼具有分层结构，其内部可观察到骨细胞，表明了具有功能的新骨形成。然而，该研究观察到的骨增量较少，这可能与实验中使用的支架性能相关。因此，有必要进一步开发hiPSC-OBs的理想生物支架[50]。值得注意的是，上述研究几乎都直接使用了原本用于诱导MSCs成骨的支架材料，因此并未将hiPSCs的存活率和二者分化过程中的差异纳入考量[51]。基于EBs法的三维体系构建有助于促进hiPSC-OBs的分化和成熟，提高细胞的功能和活性，有助于研究细胞间的相互作用和组织形成过程，因此该法多适用于骨骼发育、疾病造模及药物筛选等领域的研究[52-53]。然而，三维培养体系的建立和维护可能相对复杂，且成本较高。

EBs法虽然规避了多能性基因和长期的体外培养，但仍检测到了畸胎瘤样组织的形成[54]。同时，其体内成骨量极为有限，难以满足临床需求。直接将hiPSCs诱导为OBs的单层诱导法成为新的理想手段。该法不仅可以提高分化效率、缩短分化时间、保持细胞的增殖能力和多向分化潜能，而且只需将多能干性维持培养基更换为含有抗坏血酸（ascorbic acid，AA）、β-甘油磷酸盐和地塞米松（dexamethasone，Dex）等化学添加剂的成骨诱导培养基即可实现。本课题组利用单层法诱导hiPSCs成骨分化，在起始分化密度为80%时能获得较多的OBs[44]。单层诱导法虽然简化了操作步骤，但其分化过程仍然受到多种因素的影响，包括培养基成分、细胞接种密度、培养时间等。同样，添加生长因子也能加速hiPSCs的直接诱导成骨。Kang等[55]用腺苷处理HUES9 hESCs和IMR90p18 hiPSCs，通过作用于腺苷受体A2bR加速成骨进程。RA的加入可促进hiPSCs直接分化成骨，这可能与BMP和Wnt信号通路的激活有关[56]。生物支架材料的应用同样起着促进作用。Wang等[57]将VN多肽和成骨BFP1多肽接枝到聚多巴胺-羧甲基壳聚糖表面，促进了hiPSCs的黏附及其在成骨诱导培养基中的分化。接着，将培养表面更换为CMC水凝胶也得到了类似的结果[58]。Kargozar等[59]将hiPSCs培养在含Sr和Co的生物玻璃（bio-glass containing Sr and Co，BGs）上，表明细胞能在BGs颗粒

上黏附和扩增，并过表达成骨标志物。

尽管上述几种方法都成功实现了hiPSCs的直接成骨向诱导，但大多是将细胞接种在滋养层或基质胶表面，且诱导过程存在血清成分，导致了培养成分不明确，批次间差异大和诱导结果的不稳定性。因此，学者们基于OBs各发育阶段的研究基础，开始致力于开发成分明确条件下的分阶段式诱导方案[60]。随着对信号通路的理解更加深入，hiPSCs的成骨过程可以通过特定的小分子序列进行严格调控，从而降低分化不全、形成畸胎瘤的风险。Kanke等[38]基于Wnt、Hedgehog和BMP通路逐步诱导中胚层分化与成骨基因表达。他们在无血清和无滋养层条件下使用糖原合酶激酶3抑制剂CHIR99021和Hedgehog通路抑制剂对hiPSC进行中胚层诱导，并在成骨培养基中辅以Hedgehog受体激动剂和成骨小分子，搭建出类似于骨形成细胞自然发育过程的分阶段式诱导路径，显示Runx2、Sp7、COL1A1和骨唾液酸蛋白（bone sialoprotein，BSP）等成骨基因的显著上调。然而，这种多阶段的诱导过程存在费时费力及成本问题。Zujur等[60]进一步在明确无异种条件下，将hiPSCs分步诱导为OBs。hiPSCs先后被接种于添加了2-ME、B-27和胰岛素-转铁蛋白-硒（insulin-transferrin-selenium，ITS）的DMEM/F12液体培养基和添加了CHIR和Cyc的基本培养基的玻连蛋白（Vitronectin）修饰表面，再更换至含SAG和TH的成骨诱导培养基中培养7 d，获得骨祖细胞。CHIR和TH处理2 d后，分化为OBs。最后，在成骨诱导培养基中培养9 d，获得成熟的OBs。这种低成本、简单明确、无异种成分且混杂因素少的诱导方法是骨再生领域的一大突破（表1）。然而，多项研究表明，不同来源的hiPSCs可能会受到表观遗传的影响，从而表现出过早分化的趋势。与人成纤维细胞相比，人骨细胞来源的hiPSCs存在DNA甲基化变异，导致其形成完整细胞谱系的能力受损，即只能生成OBs和软骨细胞，而缺乏形成脂肪细胞的能力[61-62]。

综上，hiPSCs成骨向分化的最新进展可被归纳为4种策略（hiPSC-EB-MSC-OBs、hiPSC-EB-OBs、hiPSC-MSC-OBs和hiPSC-OBs），其中，基于特定化合物严格调控的分阶段式和三维定向诱导体系或逐渐成为主流（图1）。此外，在选用具体方法时，应根据实验目的、条件和资源进行综合考虑。成骨向诱导分化起始条件的研究和大规模培养可以选择生长因子分阶段诱导法，而对于需要模拟体内环境或研究细胞间相互作用的情况，可以考虑构建三维培养体系[63]。

4　hiPSC成骨分化的临床展望

在药物研发和毒性测试中，hiPSC-OBs可作为理想的体外模型，评估药物对骨形成和代谢的影响[64-66]。hiPSC-OBs还可以用于研究骨病的发病机制和病理过程，为疾病的预治提供新思路[67-68]。基因编辑技术可实现OBs的精准调控，构建具有特定功能的细胞模型，为个性化治疗提供可能[69-70]。

由创伤、疾病或手术引起的骨缺损，传统的治疗方法如自体骨移植存在供体部位疼痛、感染风险以及骨量有限等问题[71]。Kang等[55]利用腺苷诱导hiPSCs成骨的研究显示hiPSC-OBs参与了骨缺损的愈合而未形成畸胎瘤，这项研究为hiPSCs技术的运用及避免致瘤性提供了方向。hiPSC-OBs可作为种子细胞，结合生物材料构建组织工程骨，并根据缺损情况定制个性化方案，从而解决供区的局限性[72]。Ye等[73]将hiPSCs种植到盘状亲水性的丝心蛋白支架中，在体外培养条件下获得良好的多向分化潜能，用于修复成年小鼠标准颅骨缺损，5周后检测结果显示在新生骨形成方面hiPSCs移植组明显好于单纯支架组。

在骨质疏松、骨关节炎等骨病中，骨再生能力的下降是导致病情恶化的重要原因。hiPSC-OBs可通过移植或局部注射等方式，与内源性干细胞相互作用，增强病变部位的骨再生能力，改善骨微环境[74-75]。同时，hiPSCs在肌腱损伤愈合中还可协助重建血运[76]。研究表明，hiPSCs在骨关节炎早期治疗和软骨修复中具有广阔的应用前景[77]。Yamashita等[78]将纯化的hiPSCs源性透明软骨细胞微粒植入联合免疫缺陷小鼠和免疫抑制迷你猪的关节缺损处，新生软骨得以存活，并与宿主关节软骨相互融合且无肿瘤形成。同时，迷你猪体内实验的结果还表明hiPSCs可用于大型动物，预示着人类关节软骨损伤同样可被hiPSCs修复[69-70, 72, 78]。

5　前景与挑战

尽管hiPSC-OBs展现出广阔的应用前景，但其实际应用仍面临一些挑战。如，分化过程中细胞命运的精确调控、细胞移植后的存活与功能发挥、长期安全性等问题都需要进一步解决。（1）分化效率与纯度：目前的分化效率仍然有限，且尚无统一

表1　hiPSCs直接分化为成骨细胞的体系构建

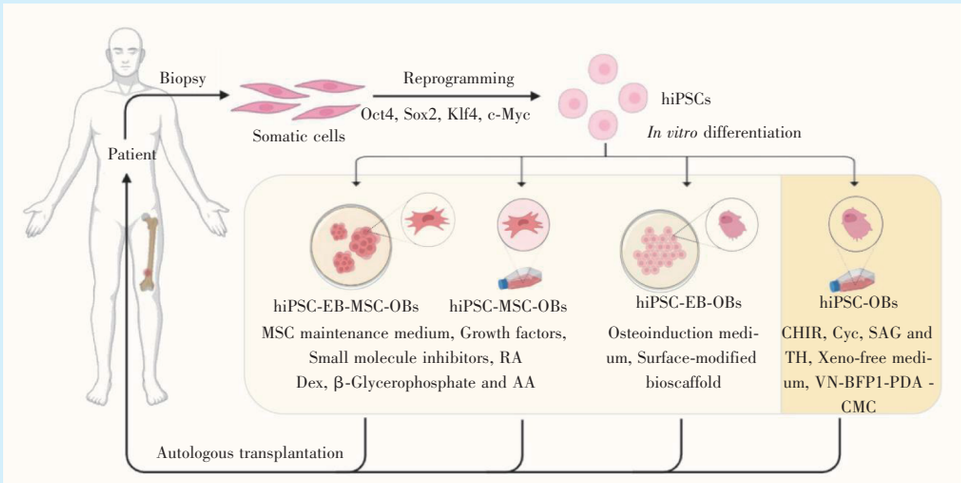
Table 1　System construction for differentiation of hiPSCs into osteoblasts

Methods	Environments and materials	Conclusion	Advantages	Disadvantages	Ref
EBs method	Osteogenesis-induced medium	Osteogenesis - induced medium can be used for direct osteogenesis toward induction of hPSCs.	●Circumvention of the pluripotency gene ●Avoidance of long-term in vitro cultures	●Formation of teratoma-like tissue ●Osteogenesis in the body is extremely limited	[43]
	Diffeasy medium (pre-induction)+matrigel surface containing Ca_2MgSi_2O7	Bioceramic $Ca_2MgSi_2O_7$ promotes osteogenesis in hiPSC - EBs.		●Direct use of scaffolds originally used to induce osteogenesis in MSCs without taking into account the survival of hiPSCs and differences in the differentiation process	[62]
	Hypoxia	Hypoxia promotes suspension and maturation of EBs and enhances adhesion and proliferative properties without compromising pluripotency.		●Differentiation, mechanism and cell purification need to be further explored	[45]
	Intermittent pressure	Intermittent pressure reduces apoptosis in EBs.			[46]
	Shear stress	Shear stress at 0.5 Pa enhances osteogenic differentiation of hiPSCs.			[47]
	PCL nanofiber scaffolds with microRNA	Functionalized bioscaffold material promotes osteogenesis in hiPSC-EBs.			[48]
Monolayer culture method	Adenosine	Growth factors accelerates osteogenesis.	●High differentiation efficiency ●Short differentiation time	●Unclear components ●Large inter-batch variations	[56]
	RA	RA promotes direct osteogenic differentiation of hiPSCs.	● Maintenance of proliferative capacity and multidirectional differentiation potential of cells	●Instability of induction results	[55]
	VN-BFP1 peptide-grafted Polydopamine - carboxymethyl chitosan surface	Surface-modified bioscaffold materials promote adhesion and osteogenic differentiation of hiPSCs.	● Simple operation: simply replace the pluripotent dry maintenance medium with the induction medium		[57]
	CMC hydrogel				[58]
	BGs particles				[59]
Defined stepwise differentiation method	Four small molecules (CHIR, Cyc, SAG and TH)	A stepwise and three - dimensional induction system with defined components is the most desirable solution at present.	●Serum-free	● The Matrigel used in this process is difficult to stabilize to maintain cellular activity	[38]
	CHIR, Cyc, SAG and TH, basal medium, osteogenesis-induced medium, Vitronectin-modified surfaces, DMEM/F12 liquid medium containing 2-ME, B-27 and ITS		●Low cost ●Clear and simple ●Xeno-free	● hiPSCs from different sources may be affected by epigenetic properties and exhibit premature differentiation ● Impaired ability to form complete cell lineages	[60]

ITS: insulin-transferrin-selenium; RA: retinoic acid; hPSCs: human pluripotent stem cells; EBs: embryoid bodies; hiPSC: human induced pluripotent stem cells; MSCs: mesenchymal stem cells; PCL: polycaprolactone

且高效的hiPSC-OBs纯化方法，使不同实验获得的细胞纯度难以保障[79]。(2)功能稳定性：hiPSC-OBs在体外分化过程中，其功能稳定性或受影响[80]。同时，细胞植入体内后的增殖、分化及成骨能力受宿主微环境影响，导致疗效的不确定性。(3)免疫排斥与安全性：虽然由患者自身体细胞重编程的hiPSCs理论上并无免疫原性，但仍有研究指出hiPSCs衍生细胞存在免疫原性差异，这可能和免疫原性抗原有关。此外，细胞在体内的长期存活、转化及致瘤风险等问题仍待进一步探讨[81-82]。(4)规模化制备与成本：hiPSC-OBs的扩增与纯化需大量人力和物力投入，导致较高规模化制备成本，限制其广泛应用。(5)法规与伦理：hiPSCs重编程操作涉及供体遗传信息，引出细胞来源的合法性、知情同意、隐私保护及生物安全等方面的顾虑[83]。

基因编辑、生物材料及生物信息学等技术不断发展，针对hiPSCs的研究将迎来更多机遇[84-85]。单细胞测序和组学技术揭示了OBs分化过程中的



Direct osteogenic differentiation of hiPSCs may become the mainstream in the future. hiPSC: human induced pluripotent stem cell; MSC: mesenchymal stem cell; OBs: osteoblasts; EB: embryoid body; Oct4, Sox2, Klf4, c-Myc: OSKM, Yamanaka four specific genes; CHIR: CHIR99021; Cyc: cyclopamine; SAG: smoothened agonist; TH: helioxanthin derivative; RA: Retinoic acid; Dex: dexamethasone; AA: ascorbic acid; VN: vitronectin; BFP1: bone-forming peptide-1; PDA: polydopamine; CMC: carboxymethyl chitosan

Figure 1　Osteogenic differentiation pathway of hiPSCs

图1　hiPSCs成骨向诱导分化路径

关键事件和调控机制，为精准调控细胞命运提供有力支持[86-87]。随着对干细胞治疗逐渐提高的社会接受度，相关法规和伦理规范也将不断完善[88]。期待通过精准调控细胞分化，实现更高效、稳定的OBs制备；通过移植方案优化，提高疗效并降低风险；通过深入探讨细胞与宿主微环境的相互作用，揭示骨再生和发病机制的更多细节，为骨病治疗带来精准、有效的手段。

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